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Title of Project

APPLICATION OF RAPID TECHNIQUES TO VIRUS CERTIFICATION IN FRUIT TREES

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3. Executive Summary

The main goal of this project was to facilitate production of virus-tested propagation material and shorten virus detection procedures for the rosaceous fruit-tree industry in Morocco and Israel. The project focused mainly on cultivars and selections of almond and apple of local interest and advantage. The targeted viruses were - the ilarviruses prunus necrotic ringspot (PNRSV); prune dwarf (PDV) and the trichovirus apple chlorotic leaf spot (CLSV).

The main research activities in this project focused on:

- Developing in vitro thermotherapy procedures and the shoot tip grafting (STG) technique for virus elimination in almond and apple hosts.
- Evaluation of rapid methods (ELISA and PCR) for determining virus status of post therapy regenerates.
- Application of target sequence amplification (PCR) techniques for detection of the targeted viruses in almond and apple cultivars.
- Technology transfer from the VC lab to the UCP group.

Thermotherapy experiments were designed with the aims of (a) improving survival rates and (b) determining the virus status of post therapy regenerates and allowing rejection of infected shoots within several weeks after the end of the heat treatment. The STG technique was adapted for almond cultivars. Selecting the optimal rootstocks, using greenhouse grown young shoots instead of in vitro grown seedling and grafting shoot tips with a meristematic dome and 3-4 leaf primordia improved the success rate of the technique.

Determining virus status was achieved by selecting sensitive ELISA assays and using PCR used to validate ELISA assays. A greenhouse indexing system based mainly on micropropagaed indicators was established and allows bioassay tests for viruses.

The results and protocols obtained in this project contributed to reliable detection and virus elimination of the targeted viruses and are relevant to other plant viruses in both countries and others.

Collaboration between the groups in Morocco and Israel has been very good and intensive, information was transferred constantly and communication very frequent. A follow up of this project is a MERC project entitled: Evaluation, mass-propagation and conservation of superior virus-free Prunus landraces, starting these days with the partners of this project (UCP and VC groups) and two additional labs.

4. Research Objectives

Production of virus-tested propagation material for an advanced fruit-tree growing industry requires reliable, sensitive technology for both virus detection and elimination.

The general goal of this project was to facilitate production of virus-tested certified propagation material and improve rapid virus detection procedures for the rosaceous fruit-tree industry of Morocco and Israel. The specific research objectives were as follows:

- Development of in vitro therapy protocols for selected almond and apple cultivars, based on:
  (a) Thermotherapy.
  (b) Shoot tip grafting.

- Development of sensitive detection assays based on:
  (a) Target sequence amplification molecular techniques (PCR).
  (b) Short-term, year-round bioassays (indexing).

The major viruses affecting stone- and pome fruit trees in Morocco and Israel– prunus necrotic ringspot (PNRSV), prune dwarf (PDV) and apple chlorotic leafspot (CLSV) were targeted.

The development of serological methods based on monoclonal antibodies and molecular methods based on target sequence amplification (polymerase chain reaction, PCR) in the last decade opened up new possibilities for diagnosis and detection of viruses and also for virus strain differentiation. Developing and validating PCR detection assays for fruit tree viruses were studied by research groups in Europe and the USA, including the Israeli group. Following the transfer of technology to Zemzami's group (UCP) in Rabat and establishing the PCR lab, this group upgraded it's diagnostic skills to a much higher level.

The development of in vitro virus elimination using thermo- and chemotherapy treatments as well as shoot tip grafting methods for woody species (including rosaceous hosts) facilitated production of virus-tested almond and apple cultivars. The Israeli group studied thermo- and chemotherapy and transferred the technology to Morocco. The Moroccan group developed the STG technique for selected almond cultivars.

5. Methods and Results

The research work performed in the frame of this project is divided into the following activities:

- Adapting in vitro methods for establishment and growing shoot cultures of -
  - virus infected almonds and apples cultivars.
  - selected stone fruit rootstocks and indicators.
- Adapting sensitive PCR assays for PNRSV, PDV and CLSV.
- Developing year-round, greenhouse indexing on woody indicators.
- Developing an efficient in vitro thermotherapy protocol for elimination of viruses in almond and apple cultivars.
- Modifying the STG technique for virus elimination in almonds.

5.1 Establishing a collection of of virus-infected stone fruits and apple cultivars and in vitro cultures

Collecting scions from virus-infected, imported and selected, local cultivars of various stone fruits and grafting them onto greenhouse grown rootstocks (mostly GF305 seedlings) was the first step in this project. Although the project focused primarily on almond and apple, other stone fruit were also collected. Establishing in vitro cultures was the second step and a pre-requisite to virus elimination studies performed in the frame of this project. Shoot cultures were established in vitro on a nutrient media. Following subcultures, the virus status was validated several times and only ELISA positive cultures served as plant material throughout this study (Table 1). This activity resulted in findings and conclusions detailed below.

5.1.1. Nutrient medium

Selecting one, basic nutrient medium for all the cultures simplified considerably the routine handling the cultures. The selected medium was the one described by Almehdi and Parfitt (1986) with minor modifications and is being used both in VC and UCP tissue cultures labs. For standard proliferation of cultures, single shoots subcultured into tubes with fresh medium yielded 3-6 axillary buds during a period of 3-4 weeks. The concentration of Benzylamino purine (BAP) was 0.5-1.0 mg /1L medium. For increased proliferation, 2mg BAP /1L medium was used.

5.1.2 In vitro clones

The source material for establishing cultures consisted of young budwood from the greenhouse or the field. Following standard surface sterilization, shoots were cut to pieces (each with 2-3 'eyes' or young buds) planted in a nutrient medium and allowed to develop. Each bud developing from an eye was considered a clone and was numbered. The propagants from each clone were maintained under the same number. During subsequent subcultures, 2 parameters were monitored (a) the proliferation factor of several clones was compared and (b) virus status as determined by ELISA.

5.1.3 Uneven virus distribution

Differences in virus levels among branches within a single tree have long been known for stone fruit trees and others. We observed a similar phenomenon when in vitro cultures were established. We observed homogeneous (ELISA-positive or ELISA-negative shoots) and heterogeneous (some ELISA-positive and some ELISA-negative) clonal populations of stone fruits but not in the apple cultures included in our study. In some clones, virus levels differed dramatically from one clone to another and in some cases ELISA-negative clones were PCR positive. For further studies in this project, only homogenous, ELISA positive populations were selected.

5.1.4 Inconsistent virus transmission

During the selection process described above, several cases of 'spontaneous recovery' of cultures occurred during the initial subcultures and plants became free of the virus (both by ELISA and PCR). This phenomenon was observed in almond cv. Kochav (54) infected with PNRSV (used as the main pollinator almond tree in Israel) and in apricot Canino infected with PDV. This phenomenon was not observed in other almond cultivars infected with PNRSV, PDV and CLSV and apple cultivars infected with CLSV. This observation emphasizes the need to monitor virus status in in vitro

virus infected collections being used for various purposes including reservoirs of positive controls, germplasm etc.

5.1.5 Selection of plant material for thermotherapy studies

Shoots selected for virus elimination studies were those with a multiplication factor of not less than 3-4 (shoots produced during the period (average of 4 weeks) between subcultures and with clearly measurable ELISA values for a given virus below (Table 1).

Table 1. A list of in vitro virus infected shoot cultures, rootstocks and indicators established in Morocco (UCP) and Israel (VC).

	Viruses			
Almond cvs.				
Chazanov	PNRSV			
Samish	PNRSV	PDV	CLSV	PNRSV + PDV PNRSV + CLSV
Um el Fachem	PNRSV			
Kochav (54)	PNRSV			
Ferraduel		PDV		
Ferranies				PNRSV + PDV
Fournat				PNRSV + PDV
Tuono	PNRSV	PDV		
Marcona				PNRSV + PDV + CLSV
Plum cvs.				
Ogden	PNRSV			
Sungold	PNRSV			
Apple cvs.				
Ana			CLSV	
24			CLSV	
Malus			CLSV	
Indicators				
GF 305				
Shirofugen				
Pyronia				
Rootstocks				
Apple MM106				
Peach x Almond 677				
Pecher de Missour (PDM)				

5.2 Standardization of ELISA and PCR assays in UCP and VC

ELISA – A joint decision was made at an early stage of the project that both labs will use one selected ELISA assay (both protocols and reagents) for each given virus. The selected assays have very specific antibodies, are sensitive and have very little or no background. For 2 viruses, the selected assays are a combination of a commercially available antibody for coating and as second antibody, monoclonal antibodies made available to us by the scientists who developed them (Table 2).

Table 2. ELISA reagents and sources used by VC and UCP

Virus	ELISA type	Reagents and source
PNRSV	DAS	IgG and conjugate, BIOREBA AG, Switzerland
PDV	TAS	IgG BIOREBA, Mab from Dr. K. Eastwell*
CLSV	TAS	IgG BIOREBA, Mab from Dr. D. Thompson**

*Washington State Univ. Prosser, WA 99350, USA. **Centre for Plant Health, Canadian Food Inspection Agency, Sidney, BC V8L 1H3, Canada.

PCR – the RT-PCR assays served throughout this project to validate ELISA assays, to detect virus in tissues with low virus levels (ELISA-negative) and in strain differentiation of PNRSV. Optimizing the PCR assays was done in VC and the technology was transferred to UCP. The PCR for CLSV was very problematic in our hands and in other labs (personal communications) despite extensive efforts to change various steps in the PCR reaction. New information on amplification of CLSV was presented in the Int. Congress in 2000 by Dr. J. Kummert (Gembloux, Belgium). Primers sequence, based on the sequence published by German et al. (1990), with 2 nt substitutions in the sense primer (comparison of different isolates) was obtained in VC from Dr. Kummert. Amplification of a PCR product, specific for CLSV, was greatly improved. Information was transferred to UCP constantly and currently both labs are using the same protocols. Details on ELISA and PCR assays for the targeted viruses are summarized below.

Table 3. PCR primer information used for RT-PCR of PNRSV, PDV and CLSV

Virus	RT-PCR Primers	Ref.	Size of product
PNRSV	5'-GAG CTC TGG TCC CAC TCA GG –3' (homologous to nt 111-130) 5'-CTG CTT GAG ATC TAG AGT GA –3' (complementary to nt 701-720)	Spiegel et al. 1996 Aparicio et al. 1999	600 bp
PDV	5'–ATG GAT GGG ATG GAT AAA ATA GT –3' (homologous to nt 1838-1860) 5'-TAG TGC AGG TTA ACC AAA AGG AT -3' (complementary to nt 1988-2010)	Parakh et al. 1995	172 bp
CLSV	5' -TTG CCA TTA TGA GGT TCA CTG G –3' (nt 5206-5227) 5' -GAT GTG AAT AGA GCC TCT CAC C–3' (complementary to nt 5576-5597 of CLSV p863)	German et al. 1990; Candresse et al. 1995	392 bp

5.3 Developing greenhouse indexing on woody indicators

The indicators selected initially were peach GF305 (the superior woody indicator for greenhouse indexing of stone fruit viruses); *Pyronia veitchii* and *P. serrulata* Shirofugen. At a later stage of the project Pecher de Missour (a almond x peach hybrid, originating from Morocco) being used as rootstock was found to exhibit symptoms following grafting infected budwood and was included also.

Micropropagation of these indicators was undertaken and they were evaluated for greenhouse indexing for the targeted viruses and others. GF305 does not root easily in vitro. The problem was overcome in the VC micropropagation lab (Dr. S. Zilkah, head) by modifying a root induction – development protocol developed by J.C. Navatel (Ctifl, France, personal communication). The induction step was done with in vitro grown shoots in a BDR medium supplemented with 1 mg/L NAA, for 7 days at 20C in the dark. Then cultures were transferred to 25C in the light. Rooting efficiency was 50-70%.

For hardening and aclimatization, rooted shoot cultures were grown in small cylinders (20 x 40 mm) containing foam polystyrene 50%, peat 40% and perlite 10% and medium, under 100% umidity for 14 days. After hardening plantlets were transferred to 200 ml pots and eventually grown under greenhouse conditions.

A simplified version of this procedure was accomplished. Green cuttings (10-12 cm long) were harvested from mother plants, disinfected in 0.5% Benlet and dipped for 20 sec. in a KIBA solution 2000 ppm. The cuttings were planted in 40 x 60 mm cylinders containing foam polystyrene 60%, peat 30% and perlite 10% and incubated in a 12/12 h. photoperiod and 100% fog humidity. The rooting efficiency using this method was 67%. Plants were vigor, grew actively and were available for grafting in the greenhouse. The 4 indicators (Table 4) are now available in UCP and VC. The rootstock Pecher de Missour was not fully explored within the time frame of this project and will be further evaluated as an indicator in the future.

Table 4. Woody indicators propagated and available at UCP and VC.

indicator	Propagation	rooting	viruses
peach GF305	seeds, in vitro	i.v. difficult	Majority of Prunus viruses
Pyronia	In vitro	easy	CLSV, ASP
Shirofugen	In vitro	easy	PNRSV, PDV,
Pecher de Missour*	seeds, in vitro	i.v. nt	To be determined

*a candidate, not yet fully evaluated to serve as an indicator.

5.4 Development of in vitro therapy protocols

Throughout this project in vitro virus elimination protocols were developed in both countries. In Morocco, shoot tip grafting (STG) was successfully developed for virus infected almond varieties. In Israel, in vitro thermotherapy was studied with virus- infected almond and apple.

In most cases hosts were infected with one of the targeted viruses. Special attention was given to almond co-infected with two or three viruses in one host. For convenience the activities are described in two parts: Shoot-tip-grafting (STG) and in vitro thermotherapy. Results were reported in the annual reports. In this final report we chose to include results and conclusions of general applicability.

5.4.1 Shoot-tip grafting (STG)

The technique involves aseptic, in vitro grafting of excised shoot-tips from infected plants onto decapitated seedling rootstocks followed by growth of the scions to develop plant material free of virus. STG has been used in certification schemes of citrus, however, for *Prunus* species an optimization of this technique was required. In the frame of this project several parameters affecting success of STG were studied with almond plants infected with the PNRSV and PDV.

- Green cuttings harvested from greenhouse-grown plants were used successfully as rootstocks for STG instead of in vitro germinated seedlings. Peach GF305 and Pecher des Missour (origin Morocco) were superior to other rootstocks. The use of green cuttings shortened and simplified considerably the traditional rootstock preparation procedure.
- Various sizes of shoot-tips excised from almond varieties were grafted in vitro and regeneration rates were determined. About 50% graft success was obtained with shoot-tips consisting of the meristematic dome and three to four leaf primordia. Since we observed a difference in the actual size (in mm) of tips originating from greenhouse plants and in vitro shoots, comparisons were made on the basis of leaf primordia numbers rather than actual size in mm.
- 'STG plants' consisting of the in vitro rootstocks and developed shoot tips (almond variety), were side-grafted in the greenhouse onto vigorous GF305 plants. Rapid growth of the grafted STG plants was observed regardless of the season.
- Virus status was first determined in side-grafted STG plants when leaf material could be collected for an ELISA / PCR assay. Budwood free of virus was allowed to grow and following repeated testing could be harvested for further propagation.

With these results, an improvement of the STG technique was obtained for almond sanitation. A schematic flow chart demonstrating the STG procedure developed in this project is enclosed in the Appendix. Results of this study were compiled and presented in the 18th International Symposium on virus & virus-like diseases of Temperate fruit crops (England, 2000) (Summary is enclosed as an Appendix).

5.4.2 In vitro thermotherapy

In vitro virus elimination has many advantages for woody hosts (e.g. *Prunus* sp.) with low tolerance to exposure, of at least several weeks, to high temperatures. Therefore, attempts were made in the frame of this project to improve thermotherapy procedures and adapt a protocol suitable for various stone fruit species and apple infected with one or more viruses. Experiments aimed to develop effective therapy protocols were studied with virus- infected almond, plum and apple with almond being the primary crop. The plant materials, viruses, types of treatments and degree of success obtained in Israel are detailed below (Table 5).

Heat treatments (temperature range 36-38°C/16 hrs light, 28-30°C/8 hrs dark, time range 21- 30 days) were applied to virus infected almond shoots grown in vitro. During the heat treatment, axillary buds were developed. The number of axillary buds depends on the level of BAP in the nutrient medium and was also a response to the high temperature stress. At the end of the heat treatment shoot tips (~ 5-8 mm long) from surviving main shoots and also from axillary buds were excised and subcultured onto a new nutrient medium for further development and testing for virus/es.

Determining the virus status of post therapy regenerates (shoots developed from the excised shoot tips) is a crucial point in deciding whether the heat-treated plant

material is free of virus or not. Several points aimed to increase the efficiency of thermotherapy were studied in this project:

- Optimizing survival of treated shoots.
- Early detection of viruses in post therapy regenerates.
- Elimination of viruses in a double-infection situation.

The effect of BAP levels on survival and proliferation

Proliferation and survival of heat treated almond shoots grown in vitro in nutrient medium with BAP ranging from 0 to 2 mg/L were compared with control shoots grown at 23C. Results of several experiments with almond shoots showed that at a BAP concentrations of 0.5 and 1 mg/L medium the proliferation rate was usually between 3 or 4 axillary buds per tube regardless of the appearance of the main shoot. Survival was above 50%. This allowed harvesting the shoot tips of the surviving main shoots and axillary buds or just the latter if the main shoot did not survive the heat treatment. Thus, the number of regenerates can be considerably large even if the survival of main shoots is relatively low.

A reliable virus detection system of post therapy regenerates

One of the advantages of in vitro virus elimination is the ability to test regrown shoots already several weeks after the end of the treatment as opposed to months (at least) in conventional therapy. The 2-3 small leaves excised from the in vitro regenerated shoots can be easily severed and tested without disturbing the growth of the shoots.

In our system the first test was done several weeks (depending on the growth rate of the treated shoots) after the excised shoot tips were placed on new medium and before their first subculture. ELISA-positives post therapy regenerates detected in the first ELISA were discarded and only the ELISA-negative shoots were subcultured. Following 2-3 further subcultures (about 3 months), shoots were subjected to a second ELISA. The number of additional positives detected in the second ELISA was low and usually no positives were detected in the third assay. ELISA-negative shoots remained free of the tested virus and no additional positives were detected in 3 subsequent tests performed over a period of about 9 months. The last test was performed following a period of storing the cultures at 4C for 4 weeks (mimicking dormancy) and several weeks of growth at 25C.

PCR assays were used (on randomly selected ELISA positive and ELISA negative samples) to validate the ELISA results and to identify low virus levels (below the threshold of ELISA). In most cases PCR results were in agreement with ELISA results. In a few cases, samples testing negative in the 1st ELISA were positive in a PCR assay, but tested positive in the 2nd ELISA, indicating a low virus level which increased later. The detection system we adapted to our experimental system allowed rejecting the majority of infected shoot cultures a few weeks after the end of the heat treatment and minimizing unnecessary subcultures.

Virus elimination in almond shoots with double infections

Initially, we intended to study in vitro thermotherapy in almond with two double infection systems. (1) cv. Samish infected with PNRSV + CLSV (belonging to different virus groups) and (2) cv. Fournat infected with PNRSV + PDV (belonging to the same virus group and closely related). Following the first heat treatments, we observed a very poor survival of Fournat shoot cultures during the heat treatment. This may be due either to heat sensitivity of this cultivar or to endophytic bacteria

(obvious from the turbidity of the medium). These results stress the importance of a reliable STG procedure for Fournat and other cultivars with similar problems.

Results and conclusions are based only on experiments with cv. Samish.

- Comparing the virus levels in shoots infected with one virus and both viruses showed that the PNRSV infected shoot population was quite homogenous. A405 values ranged from 0.4 – 0.8 O.D. (single infection) and 0 – 0.8 O.D. in a double infection. The CLSV infected shoot population was heterogeneous. A405 values ranged from 0-1.0 both in single and double infections. In the latter values were relatively higher than in the single infection (Fig. 1 in Appendix).
- Survival of Samish shoots co-infected with PNRSV + CLSV was similar to those infected with one of the virus.
- Elimination of PNRSV in response to a heat treatment of 36/28C (alternating during day and night) was achieved when shoots were exposed to this regime for at least 4 weeks. This treatment did not eliminate CLSV.
- Elimination of CLSV was obtained only when temperature was raised to 38C and the treatment prolonged to 51/2-6 weeks.
- Testing post therapy regenerates showed that a high percentage of the total PNRSV-positive shoots (cumulative results of 4 subsequent assays) were detected in the 1st assay. For CLSV, the percentage of total positives was similar in assays 1 and 2. No difference was observed in detection of viruses in MS and Abs.

Based on these results it was concluded that elimination of viruses in a double infection requires tailoring a suitable thermotherapy treatment or alternatively using the STG technique.

Table 5. Plant materials, viruses and treatments in virus elimination experiments

Plant material	Virus	Treatment	Degree of success***
Almond		Heat treatment* 21 days	+
Cv. Samish, Chazanov	PNRSV	28 days	++
Um el Fachem,	PNRSV	28 days	+
Kochav (54)	PNRSV	spontaneous virus free buds	
Fournat	PNRSV + PDV		-
Plum		Heat treatment 28 days	+
Cv. Ogden	PNRSV	Chemotherapy** (virazole) 28 days	-
		Combined heat + virazole 28 days	++
Sungold	PNRSV	Heat treatment 28 days	++
Apple		Heat treatment 28 days	-
Cv. Ana,	CLSV	Chemotherapy (virazole) 28 days	+
Rootstock Malus 70		Combined heat + virazole 28 days	++

*heat treatment includes exposure to 36-38C for 16 hrs light followed by 28-29C for 8 hrs dark.

**virazole 20 mg/L is included in the medium.

***evaluated as number of virus free regenerates. – ineffective; + effective; ++ very effective.

6. Impact Relevance and Technology Transfer

Benefits of UCP from this project are numerous. They included in particular:

1. Improvement of virus elimination by thermotherapy and STG in almond varieties
STG was applied successfully to sanitation of almonds and permitted recovery of virus-free almond plants from various varieties with single -and double- infected with the viruses *Prunus* necrotic ringspot (PNRSV) and prune dwarf (PDV). Results of this work were presented in the 18th International Symposium on virus & virus-like diseases of Temperate fruit crops (England, 2000) and published in the proceedings.

Table 6. Sanitized virus-free almond varieties available at UCP repository

Variety	Sanitation technique	Viruses eliminated
Ferraduel	TT/STG	PDV
Ferranies	TT/STG	PNRSV + PDV
Fournat	TT/STG	PNRSV + PDV
Marcona	TT/STG	PNRSV + PDV + CLSV
Tuono	TT/STG	PNRSV
Tuono	TT/STG	PDV

TT/STG thermotherapy treatment (35°C/16 hrs light, 30°C/8 hrs dark, for 14 days) was applied to in vitro shoot cultures prior to excising shoot tips and performing the shoot tip grafting method.

Other local selections of almond of local origin were collected from remote areas of South Atlas mountains have been established under greenhouse and undergo a sanitary evaluation. The root-stocks evaluated within the scope of this project include GF305, peach GF677, Pecher de Missour, prune Myrobolan and cherry Shirofugen. Clean material from these varieties and rootstocks is maintained under insect-proof plastic house and supplied to nurseries.

2. Application of in vitro virus elimination methods to apples
Tissue culture of apples was never attempted in Morocco. After training in tissue culture in Volcani Center, initiation of apple culture from local varieties was undertaken at UCP facility.
The difficulties associated with explant oxidation were overcome and application of tissue culture sanitation techniques became possible for the first time in Morocco. Hence, in vitro cultures from local varieties (Golden Smooth, Red Chief x Starck) and root-stock (MM106) are now maintained in the UCP facility.

3. Technology transfer
The personnel of UCP was trained at the Volcani Center on diagnostic and tissue culture techniques. Acquired know-how along with assistance provided throughout expertise visits of the Israeli partners to Morocco and persistent communication enabled us to develop and improve diagnostic techniques for reliable and quick detection of PNRSV, PDV and CLSV. RT-PCR detection of these viruses was applied for the first time in Morocco within this collaboration.

4. Scientific exchange and development of friendship
A large group of scientists from the Volcani including 5 professors was invited to

Morocco and presented Seminars to a large Auditorium on up to date subjects of interests to agriculture in the Mediterranean region.
This led to establishment of lasting academic, professional relation and friendship unprecedented between Israel and any other Arabic country.

7. Project Activities/outputs

Meetings attended and held during the project
Moroccan and Israeli scientists cooperating in this project met at least once a year using funds from this project and other sources. The visits included - reviewing results, sharing and experiencing lab protocols, discussions and planning future work Field trips to commercial and experimental orchards, indexing plots, nurseries and plant collections were included.
1998 – Ing. Rizqi attended the 17th International symposium on Virus and Virus-Like Diseases of Temperate Crops in Beltsville, MD, U.S.A. (July 1998). He had the opportunity to visit with the Israeli partners for planning of the activities. He also established contacts with leading scientists in fruit tree virology.
Dr. Spiegel visited Morocco in December 98 to review the progress of the project, visit the facilities in place and demonstrate some techniques for the Moroccan team.
1999 - Ing. M. Zemzami and Dr. M. Aaouine visited Spiegel's lab in Feb. 99. The visit included discussions and future plans in the project, field trips to the south and north, a meeting with Mr. W.H. Crane and B. Ayalon of the US Embassy in Tel Aviv and a meeting of the four partners for the MERC pre-proposal in Rahan Meristems lab in kibutz Rosh Hanikra.
S. Spiegel visited Morocco in April 99. The progress of the project was evaluated with M. Zemzami, A. Rizqi and others and various problems were raised and discussed.
In mid-July 99, towards the final compilation of the MERC proposal, Ing. Zemzami and Dr. Aaouine (using funds from their own budgets) spent several intensive days with the Israeli partners in Rosh Hanikra, hosted by Rahan Meristem.
Ing. A. Rizqi attended the International Plant Protection Congress in Jerusalem. He later spent 2 weeks in Spiegel's lab getting 'hands-on' experience in tissue culture techniques and visiting with numerous scientists in the Institute of Plant Protection at VC.
2000 - S. Spiegel and A. Rosner visited UCP, Morocco (April and May respectively). Dr. Rosner's stay was devoted mainly to working together with the UCP team on PCR techniques and overcoming various problems.
During Spiegel's visit, the progress of the project was evaluated with M. Zemzami, A. Rizqi and others. An outline for a research paper (A. Rizqi, senior author) was prepared.
During May 2000, Dr. Ibtissam Alamy and Ms. Nadia Atif (UCP group), both involved in this project, spent two weeks training in Spiegel's lab. Their time was devoted to gaining experience in vitro thermotherapy procedures, PCR, ELISA, micropropagation of rosaceous plants, rooting and hardening. Visits to other ARS labs in and out of the Volcani Center, stone fruit orchards, a major nursery, fruit packing-house etc. were included. It is expected that this visit will be beneficial for the in vitro virus elimination system in UCP.

Public Presentations

- In April 99, several scientists of the Virology Dept (funds from other sources) joined S. Spiegel towards the end of her visit in Morocco. A one-day meeting (open to the public) was organized in Rabat by UCP and DPVCTRF and hosted by the latter. Dr. Lakhdar (PDVCTRF) chaired the morning session. Ing. Zemzami presented an overview of the project and Drs. Spiegel, Raccah and Gera gave seminars on their current research work. Ing. Zemzami translated from English to French. An active discussion followed the presentations. Attendance ranged from 65 – 150 persons during the day (program of seminars enclosed).
- During his visit in Spiegel's lab (July 99), Mr. Rizqi gave a seminar and a live demonstration (open to VC scientists) of the STG technique modified by him for stone fruit species.
- In July 2000 Dr. Spiegel presented (on behalf of both research group) a paper entitled 'Recovery of virus-free almond plants by improved in vitro shoot-tip grafting. (A. Rizqi, senior author) in the 18th International Symosium on Virus & Virus-like Diseases of Temperate Fruit Crops, held in England.

Publications

S. Spiegel, A. Rosner, Y. Tam, S. Zilkah, E. Faingersh, A. Rotbaum and L. Krizbai 1999. Detection of prune dwarf virus in sweet cherry in Israel. Acta Hortic. 472:249-255. Proceedings of the 17th International Symposium on Virus and Virus-Like Diseases of Temperate Crops. ed. A. Hadidi.

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8. Project Productivity

Both the Moroccan and Israeli scientists involved in this project concluded that the project was very successful and contributed to improving fruit tree growing in the two countries and to plant virus detection. The decision to include the STG technique in

the planned research work in Morocco contributed to the development of this technique for almonds and is of great benefit to sanitation of almonds.
The technology transfer, training and on going information exchange between the groups contributed to its success.

9. Future Work

The two groups involved in this project and two additional groups - Domaines Agricole El Basatine (Dr. M. Aaouine, Head) and Rahan Meristem (Dr. E. Khayat, scientific director) are about to start a MERC project entitled: "Evaluation, mass-propagation and conservation of superior virus-free Prunus landraces". The partners are looking forward to joint effort and extensive continued collaboration.

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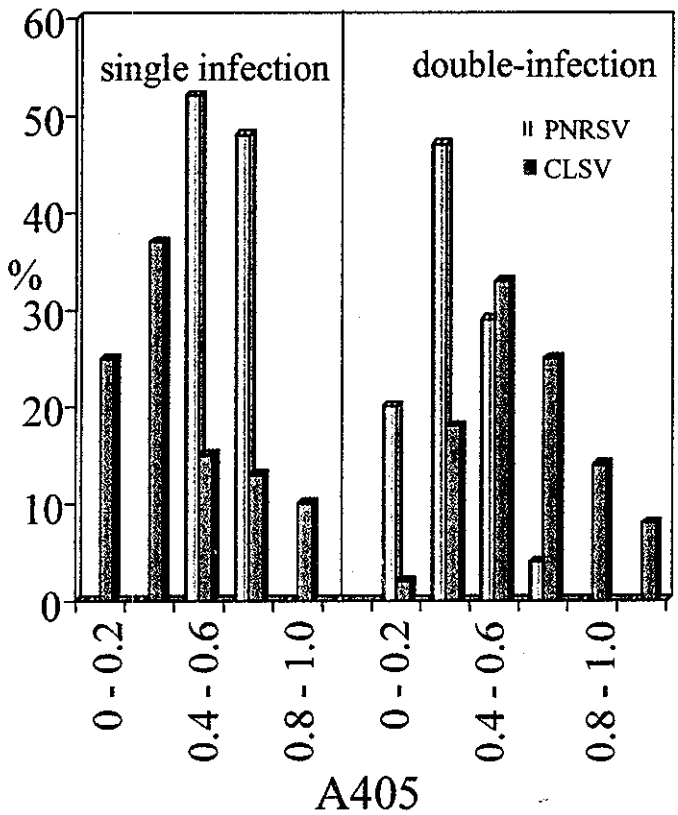
Appendix

- Fig. 1 PNRSV and CLSV levels, measured by ELISA in single infected and co-infected almond Samish shoots.
- First page of STG publication.
- Schematic description of the shoot tip grafting procedure.
- *In vitro virus elimination scheme.*
- Title page of MERC project
- Photographic documentation of visits and STG on cover-page of the Proceedings.

Fig. 1. PNRSV and CLSV levels, measured by ELISA in single infected and co-infected almond Samish shoots.



In vitro grown almond shoots infected with PNRSV (single infection and with PNRSV+CLSV (double infection), were tested for virus by ELISA. A405 values (0-1.0) were divided into groups. 50 shoots were used for each test.

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RECOVERY OF VIRUS-FREE ALMOND PLANTS BY IMPROVED IN VITRO SHOOT-TIP GRAFTING

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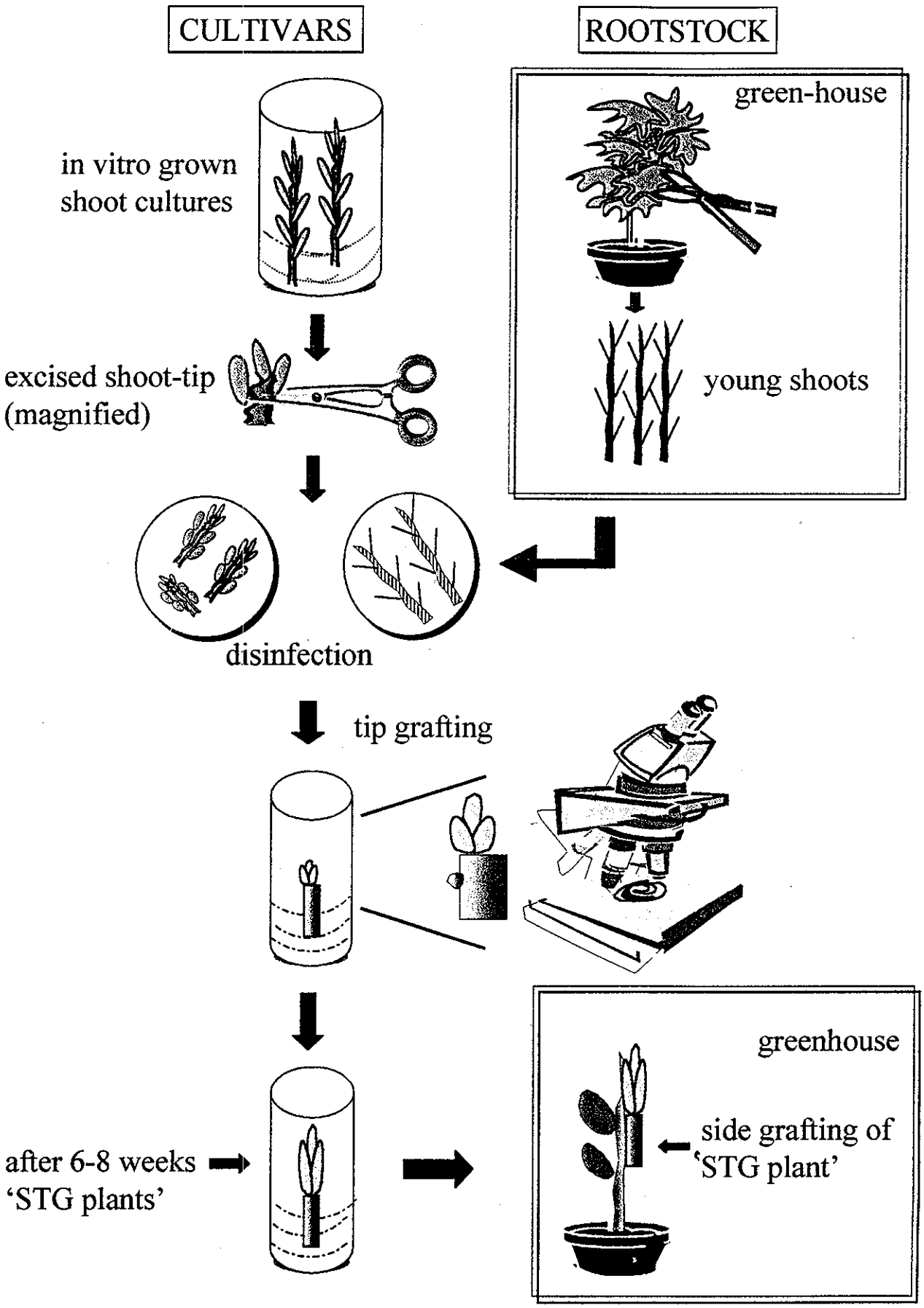
Keywords: PNRSV, PDV, virus elimination

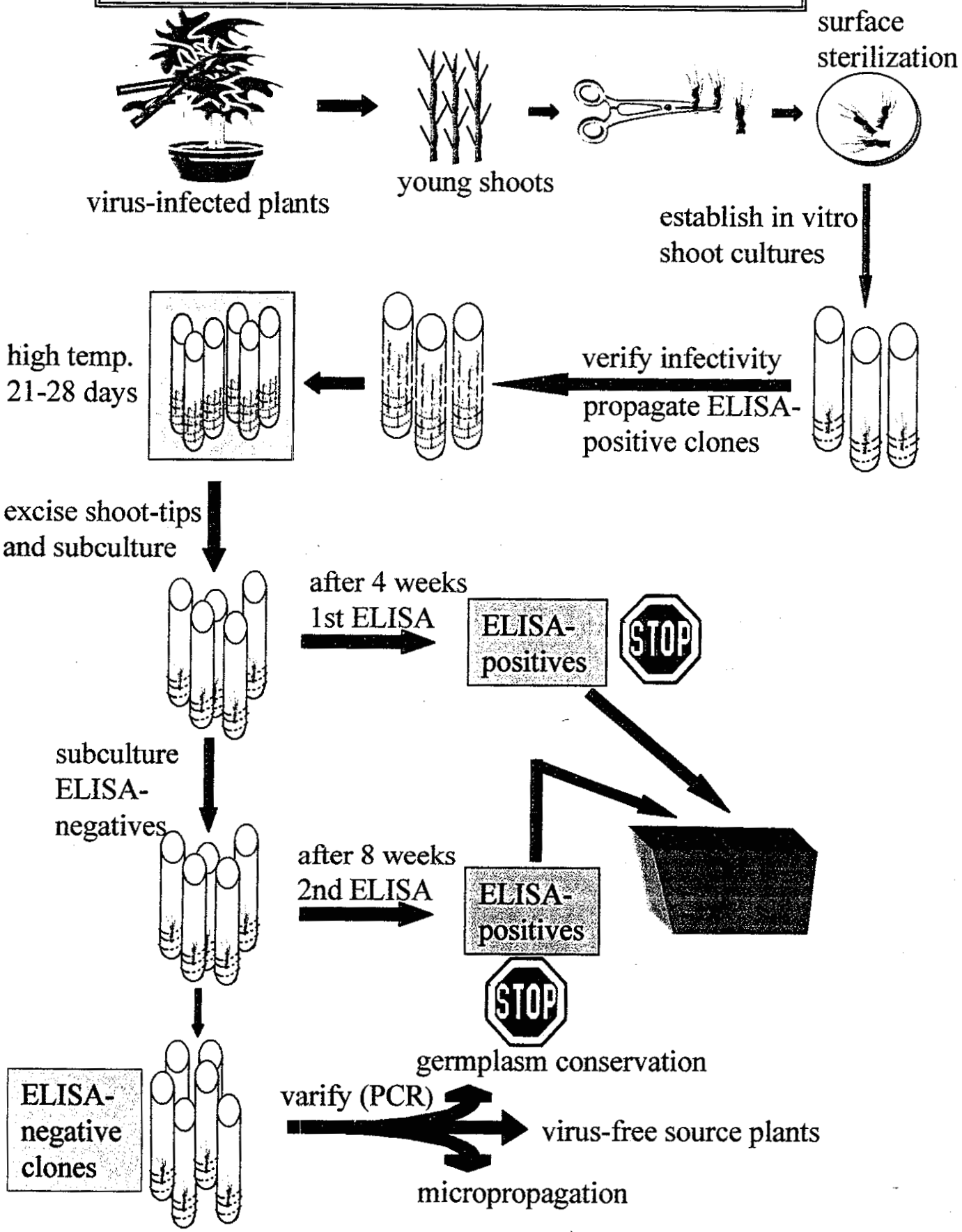
Abstract

Shoot-tip grafting (STG) involves aseptic, in vitro grafting of excised shoot-tips from infected plants onto decapitated seedling rootstocks followed by growth of the scions to develop plant material free of virus. STG has been used in certification schemes of citrus, however, for *Prunus* species an optimization of this technique is required. In the present study, several parameters affecting success of STG were studied with almond plants infected with the viruses prunus necrotic ringspot (PNRSV) and prune dwarf (PDV).
- Green cuttings harvested from greenhouse-grown plants were used successfully as rootstocks for STG instead of in vitro germinated seedlings. Peach GF305 and pecher des Missour (origin Morocco) were superior to other rootstocks. The use of green cuttings shortened and simplified considerably the traditional rootstock preparation procedure.
- Various sizes of shoot-tips excised from almond varieties were grafted in vitro and regeneration rates were determined. About 50% graft success was obtained with shoot-tips consisting of a meristematic dome and three to four leaf primordia.
- 'STG plants' consisting of the in vitro rootstocks – developed shoot tips (almond variety), were side-grafted in the greenhouse onto vigorous GF305 plants. Rapid growth of the grafted STG plants was observed regardless of the season.
- Virus status was first determined in side-grafted STG plants when leaf material could be collected for an ELISA / PCR assay. Budwood free of virus was allowed to grow and following repeated testing could be harvested for further propagation.
With these results, an improvement of the STG technique was obtained for almond sanitation. The study is ongoing and its application for other *Prunus* species will be attempted.



schematic description of the shoot tip grafting procedure

IN VITRO VIRUS ELIMINATION SCHEME



S. Spiegel, 2000

USAID – THE MIDDLE EAST REGIONAL COOPERATION PROGRAM [MERC]

a. Title:
Evaluation, mass-propagation and conservation of superior virus-free *Prunus* landraces

b. Submitting Institution:
Agricultural Research Organization (ARO), The Volcani Center
Bet Dagan, 50250, Israel

c. Proposal Identification Number: M20-072

d. Investigators:
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Project leader: SPIEGEL, S.
Principal Co-investigator: ZEMZAMI, M.

f. Amount of total requested funds: (US$) 1,000,000
g. Duration: 4 years

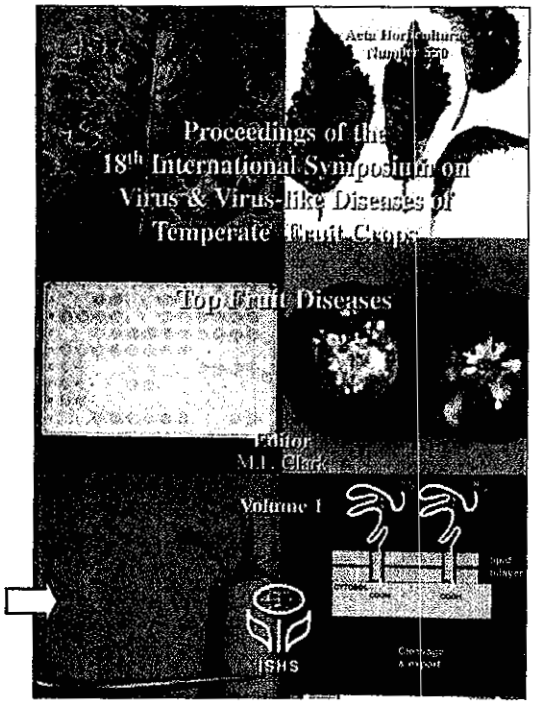
III. TECHNICAL SUMMARY

The overall aim of this project is to advance the stone-fruit growing industry in Morocco and Israel by facilitating production of certified propagation material of locally selected superior rootstocks and cultivars. To this end, the following major objectives are proposed: (a) selection of almond genotypes from various regions of Morocco and evaluation for drought and salinity tolerance, (b) developing *in vitro* and /or *in vivo* rooting and propagation methods for mature almond idiotypes, (c) development and application of *in vitro* virus elimination methods for stone fruit rootstocks and cultivars, aimed to shorten the production of virus-tested plants, (d) development and validation of improved PCR techniques allowing detection of viral pathogens in post therapy shoot cultures, (e) optimization of tissue culture protocols for mass propagation of the selected local plant material, (f) establishment of cryopreserved and *in vitro* maintained germplasm collections of selected Moroccan almond genotypes. The project will focus on the four stone fruit crops: almond, plum, peach and appricot. The targeted pathogens are three major viruses and one viroid.

The expected selection and propagation of rootstocks tolerant to abiotic stress conditions and adapted cultivars, combined with effective virus elimination and detection techniques, will strengthen the much needed, local production of virus-tested stone fruit planting material and facilitate safe exchange between Morocco, Israel and other countries. The increased use of such plants will translate into higher income for farmers, thus improving the economic status of communities in rural areas.

Visit of tissue culture facility in El Basatine. From left to right: Dr. Sara Spiegel, Dr. M. Aaouine, Ing. M. Zemzami (Dec. 99).

Visit of the tissue culture lab, VC, Bet Dagan. From left to right Dr. Ibtissam Alamy, Mrs. Yehudit Tam, Ms. Nadia Atif (May 2000)



STG developed in UCP on the cover-page of the Proceedings volume. (2001).

Discussing results and planning the MERC proposal. in VC. From left to right: Ing. M. Zemzami, Dr. M. Aaouine, Dr. S. Spiegel, Dr. A. Rosner. (Feb.1999)

Discussing results in UCP. From left to right: Dr. S. Spiegel, Ing. M. Zemzami, Ing. A. Rizqi.(April 2000)